ISOLATION-AMPLIFICATION OF *ENV-TM* SUBUNIT GENES OF JEMBRANA DISEASE VIRUS BY A SINGLE STEP RT-PCR AND ITS DIRECT CLONING IN PCR2.1-TOPO PLASMID

Asmarani Kusumawati[1,2], Basuki Hidayat[2], B. Sardjono[1] and Joannes Sri Widada[3]

ABSTRACT

Jembrana virus is agent of infectious Jembrana disease virus (JDV) in *Bos javanicus*. The genome of Jembrana virus is a single-stranded RNA of 7,732 nucleotides in length. In this experiment, the isolation of *env-tm* subunit gene of Env protein from the viral genome by a single-step RT-PCR reaction. RT-PCR products were directly cloned in PCR2.1-TOPO plasmid by topoisomerase based-TOPO TA cloning kit. With this system, prior purification and restriction enzyme digestion of DNA fragments are no more required. A high number of positive recombinant bacterial colonies were obtained which were further analysis by *Bam*HI digestion for a definite characterization of positive clones. Topoisomerase based cloning procedure is highly efficient and allows a rapid and easy gene isolation.

(Key words: Jembrana virus, *env-tm* gene, Topoisomerase cloning).

Buletin Peternakan 27 (1) : 1 - 7, 2003

[1] Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia.
[2] Center for Biotechnological Study, Gadjah Mada University, Yogyakarta, Indonesia.
[3] Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia.

AMPLIFIKASI-ISOLASI GEN ENV SUBUNIT TM DARI VIRUA PENYAKIT JEMBRANA DENGAN SATU STEP RT-PCR DAN KLONING GEN PADA PLASMID PCR2.1-TOPO

INTISARI

Virus jembrana adalah agen infeksius penyakit jembrana pada *Bos javanicus*. Genom virus jembrana adalah satu RNA rantai tunggal (ssRNA) dengan panjang 7.732 nukleotida. Telah dilakukan isolasi gen *env* subunit TM dari protein ENV dari genom viral dengan reaksi satu step RT-PCR. Produk RT-PCR langsung diklon pada plasmid PCR2.1-TOPO dengan didasarkan pada sistem topoisomerase dengan menggunakan kit TOPO TA cloning. Pada sistem ini langkah purifikasi dan digesti fragment DNA menggunakan enzim restriksi ditiadakan. Koloni bakteri protein rekombinan dihasilkan sangat banyak. Analisis selanjutnya adalah digesti dengan *Bam*HI untuk memastikan karakteristik klon positif. Isolasi gen dengan prosedure kloning berdasarkan sistem Topoisomerase memberikan hasil dengan efisiensi yang tinggi, cepat dan mudah.

(Kata kunci : Virus Jembrana, Gen *env* subunit TM, Topoisomerase cloning)

Introduction

Jembrana disease virus (JDV) is the agent of an acute infectious Jembrana disease (JD) of Bali cattle (*Bos javanicus*), reported for the first time in Jembrana village in Bali, Indonesia (Wilcox *et al*., 1992). JD is not unique to *Bos javanicus*. Other types of cattle are also susceptible, namely Friesian (*Bos taurus*) and crossbred Bali (*Bos javanicus* x *Bos indicus*) cattle although the resulting lesions are milder when compared to a case fatality rate of about 20% in *Bos javanicus* (Soeharsono *et al*., 1995a). During the acute phase viral particles can be detected in saliva and milk and the titer of infectious virus in blood is high. Direct transmission of the disease occurs by the conjunctival, intranasal or oral routes (Soeharsono *et al*., 1995b). In experimentally infected animals, many JDV-infected tissues were demonstrated early in the disease course. The most infected organ is spleen but other organs are also highly infected, i.e. lymph nodes, lungs, bone marrow, liver and kidney (Chadwick *et al*., 1998).

Antigenic cross-reactivity between elements of JDV with the previously identified bovine lentivirus designated bovine immunodeficiency virus (BIV) suggests that JDV is a lentivirus (Wilcox *et al*., 1995). The sequenced JDV genome consists of a single-stranded RNA, 7,732 nucleotides in length (Chadwick *et al*., 1995b). Sequence comparison established that JDV is a lentivirus (Wilcox *et al*., 1995a, 1995b). Though closely related, significant genomic differences were found between JDV and BIV which may be related to the differences in pathogenicity between these two viruses (Chadwick *et al*., 1995b). JDV has been reported to be also related to human immunodeficiency virus (HIV) and some of its regulatory elements can functionally substitute for those of HIV (Chen *et al*., 2000) (Metharom *et al*., 2000).

Env protein is one of the structural protein of JDV. It is composed of two subunit i.e. surface subunit (su) and transmembrane subunit (tm). In this experiment, the isolation and amplification of *env-tm* gene by an RT-PCR single reaction tube and the direct cloning of the amplification products in pCR2.1-TOPO by a topoisomerase based-methodology was correct out.

Materials and Methods

Isolation of total RNAs

Total RNAs were isolated from lymph nodes of infected animal using TRIZOL reagent (Life Technologies), according to the manufacturer recommendation. Briefly, approximately 70 mg of tissue were homogenized in 1 mL of TriZol, then allowed for 10 minutes at room temperature. After centrifugation at 12,000 rpm, for 10 minutes in a bench minicentrifuge, the supernatant was separated and treated with 0.2 mL chloroform then precipitated with 0.5 mL isopropanol. The pellet was washed with 70% ethanol and dissolved in 100 µL of steril DEPC-treated water. The purity and concentration of the RNA preparation were spectrophotometrically established.

Purification of viral RNA

Viral particles, isolated by sucrose gradient, were incubated for several minutesutes at room temperature in 10 mM Tris-HCl pH 7.5 and 0.4% SDS. RNA was purified by a combination of phenol and chloroform treatment. RNA was precipitated with isopropanol and washed with 70% ethanol, then dissolved in steril DEPC-treated water. purity and concentration of RNA were spectrophotometrically determined.

Isolation and amplification of env-tm gene

Env-tm gene was isolated and amplified from total RNAs or purified viral RNA using Titan One Tube RT-PCR kit (Roche), according to the instruction manual provided. This kit allows the reverse transcription of RNA and the PCR amplification in a single reaction tube. The primers used, derived from a published sequence (Chadwick *et al.*, 1995b), were described in Results and Discussion. RT-PCR reactions were performed in 50 µL of buffer, containing 0.4 µM of each primer, 1 µL enzyme mix (reverse transcriptase and polymerases) and variable amounts of RNA, with the following conditions: reverse transcription at 50°C for 30 minutes; denaturation at 94°C for 2 minutes; 35 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, and elongation at 68°C for 1 minutes 30 seconds; finally an additional elongation step at 68°C for 10 minutes then 4°C. After RT-PCR completion, 1.0 unit of Taq polymerase was added and the reaction continued for 10 minutes at 72°C.

Cloning in PCR2.1-TOPO

Cloning in PCR2.1-TOPO plasmid was carried out using TOPO TA Cloning system (Invitrogen), according to the instruction manual. This cloning methodology, based on the topoisomerase system, enables cloning of exogeneous DNAs by direct use of amplification products. Topoisomerase reaction was done in 6 µL, containing 1 to 2 µL of the RT-PCR reaction products and 1 µL TOPO vector, for 5 minutes at 22°C. Reaction products were kept on ice or at -20°C before use.

Transfection in E. coli DH 5α

Transformation was done using TSS method in *E. coli* DH 5α. TSS-competent *E. coli* DH 5α bacteria were obtained by 10 times-concentrating fresh exponential phase bacterial culture (OD_{600}nm around 0.6) in LB containing 10% PEG 6,000 (w/v), 5% DMSO (v/v) and 35 mM $MgCl_2$. Different amounts of the transformation were spread on LB agar plate containing 50 µg/mL amphicillin, 40 µL of 40 mg/mL X-Gal and 40 µL of 100 mM IPTG and incubated at 37°C, for one night (OVN). White bacterial colonies were cultured in 5 ml LB-amphicilline for OVN. Bacteria were harvested by centrifugation (Sorvall, 4,000 rpm, 10-15 minutes, 4°C). The bacterial pellet was used for plasmid preparation.

Minutesi-preparation of plasmid

Plasmid preparation was done using alkaline lysis method. Briefly, pelleted bacteria was dissolved in 0.3 ml buffer 1 (50 mM Tris-HCl, pH 7.5, 10 mM EDTA) and incubated for 5 minutes at room temperature. 0.3 mL of buffer 2 (0.2 M NaOH, 1% SDS)

was then added and the solution was mixed without vortexing. Finally 0.3 mL of buffer 3 (2.55 M potassium acetate, pH 4.8) was added and the solution mixed without vortexing then centrifuged in a minicentrifuge for 15 minutes at maximum speed (13,000 rpm) at room temperature. The supernatant (0.8 mL) was then precipitated by addition of 0.7 mL isopropanol and centrifugation (minicentrifuge, 13,000 rpm, 15 minutes, room temperature). The pellet was washed with 70% ethanol and slightly dried.

Analysis of clones

Plasmid pellet was dissolved in 50 µL 10 mM Tris-HCl pH 7.5 and 0.5 mg/mL RNAses and incubated at 37°C for 30 minutes. Plasmid analysis was carried out by double digestion of 2.5-5.0 µL plasmid solution with *Bam*HI, in 20 µL buffer, 5 units of each enzyme, at 37°C for 1 h 30 minutes. Digestion products were then analyzed by electrophoresis on a 0.8% agarose gel. Revelation was done by ethidium bromide and observation under UV lamp.

Results and Discussion

Conception of primers

The primers, derived from a published sequence (Chadwick *et al*., 1995b), used in the RT-PCR reactions are as follows: a) upstream primer: ATAGGATCC**ATG**GCCGTGGGG-ATGGTCATAT (initiation codon **ATG** in bold letters, introduced *Bam*HI site underlined); b) downstream primer CAGCGGATCCTCCAAGCTACGTGTC (introduced *Bam*HI site underlined). The introduced restriction sites are intended for easy gene excision and correct further cloning in expression vectors while the initiation codon is aimed for correct protein synthesis in eukaryiotic cells. With these primers, the entire coding sequence of *env-tm* will be isolated and amplified, including the highly hydrophobic C-terminal region.

Isolation and amplification of env-tm gene

Env is one of the structural proteins of JVD, the agent of severe infectious disease of *Bos javanicus*. It is composed of 2 subunits i.e. surface (su) and transmembrane (tm) subunits. We present in this paper the isolation and amplification of the entire coding sequences of *env-tm*, about 1.1 kb in length by RT-PCR reaction as the genome of Jembrana virus is constituted of a single-stranded RNA molecule. Till recently, in nucleic acid amplification procedures, reverse transcription had to be performed prior to amplification. The procedures require much longer time. Due to Rnase sensibility of RNAs, a high amount of initial materials would be needed. The availability of one step RT-PCR kits that we used, allows reverse transcription and amplification reactions in a single reaction step. Much shorter times are therefore needed. The kit we used contains a mix of AMV reverse transcriptase, Taq DNA polymerase, and a proofreading polymerase.

RT-PCR as described in Materials and Methods, was carried out using total RNAs or isolated viral RNA as matrix. A Taq DNA polymerase treatment was added to complete the amplification process. Figure 1 shows the analysis of the resulting DNA fragments. The length of the DNA fragment corresponded to the expected *env-tm* gene lenght, i.e. *circa* 1.1 kb. RT-PCR reactions, performed with a kit from Roche, already results in DNA fragments with single 3' adenine overhang though not in all the amplified fragments. The additional Taq polymerase treatment was thus aimed to increase the extents of this 3' adenine overhang intended for cloning optimalization in pCR2.1-TOPO plasmid, using TOPO TA cloning system (see below).

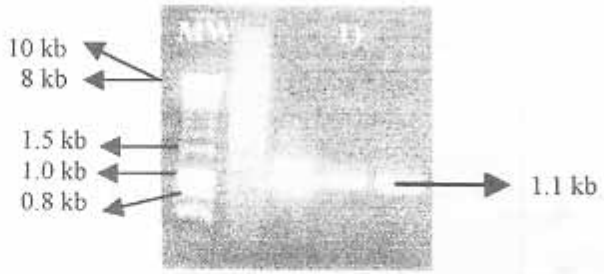

Figure 1. Analysis of *env-tm* gene isolation/amplification by RT-PCR reaction.

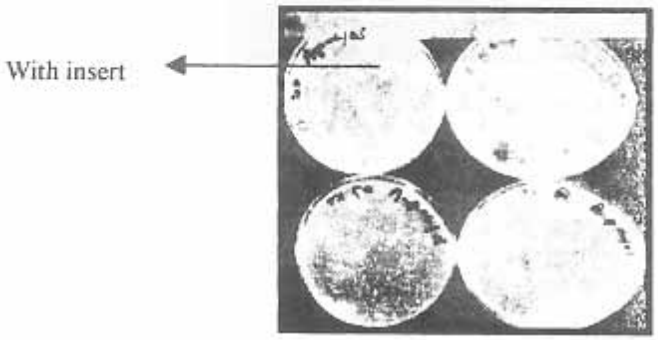

Figure 2. Transfection in *E. coli* DH 5α.

RT-PCR reaction was done as described in Materials and Methods. One fifth of the reaction products was analyzed by electrophoresis on a 0.8% agarose gel. 1) :total RNAs or viral RNA as matrix; MW: molecular weight markers.

Result of transfection in E. coli DH 5α

Cloning in pCR2.1 vector was done using TOPO TA cloning kit. This system enabled direct cloning of the amplification products resulting from RT-PCR or PCR reactions. DNA purification or restriction enzyme digestion was no more needed. Therefore, it could accelerate the cloning process. The system was based on topoisomerase reaction for gene insertion in plasmid, instead of ligation with DNA ligase. After the topoisomerase reaction, the resulting products were transfected in *E. coli* DH 5α using TSS method. Figure 2 shows the ampicilline-resistant bacterial colonies obtained.

In pCR2.1-TOPO, insertion of exogeneous DNA takes place in galactosidase gene. It will therefore interrupt the gene hence the production of non active enzyme. The presence of recombinant plasmid in bacteria will result in amphicilline-resistant recombinant white or light blue bacterial colonies. Ampicilline-resistant blue colonies are so bacterial transformants harvesting pCR2.1-TOPO plasmid devoid of insert.

Due to topoisomerase, the single 5' thymidine overhang of the vector and the 3' adenine overhang of the RT-PCR/PCR products, insertion of exogeneous DNA fragments is highly enhanced when compared to the classical insertion by DNA ligase. This resulted in obtaining a very high number of positive bacterial transformants (Fig. 2).

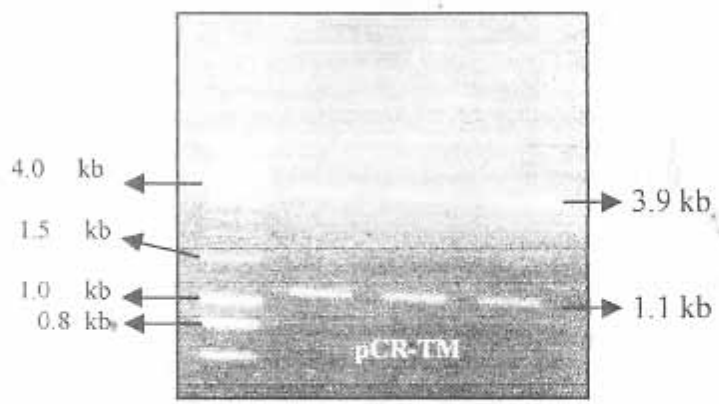

Figure 3. Clone analysis by *Bam*HI digestion.

E. coli DH 5α was transfected with the topoisomerase reaction products by the TSS method. Bacteria were spread on LB-amphicilline-agar plate containing IPTG and X-Gal.

Clone analysis and obtaining pCR-TM plasmid

In pCR2.1-TOPO-based construction, positive clones can already be determinutesed by the coloration of the bacterial colonies, as dicussed above. Nevertheless white or light blue bacterial colonies may also result from a frame shift of codons. Therefore to establish definitely that white or light blue bacterial colonies actually harvest plasmid containing *env-tm* gene, further analysis was carried out by a digestion with *Bam*HI. Plasmids were prepared from white bacterial colonies as described in Materials and Methods. Analysis results by *Bam*HI digestion are given in Figure 3. Positive recombinant plasmids will give rise to two fragments of 3.9 kb (vector) and of *circa* 1.1 kb (*env-tm* gene) respectively. Figure 3 clearly shows that we actually obtained positive recombinant plasmid, i.e. pCR2.1-TOPO plasmid containing *env-tm* gene, which we called pCR-TM.

Digestion was performed as described in Materials and Methods and the digestion products were analyzed by electrophoresis on a 0.8% agarose gel.

Cloning in pCR2.1-TOPO plasmid, performed with TOPO TA Cloning system from Invitrogen, takes advantage of the topoisomerase reaction for easy, rapid and efficient gene insertion and amplification. This process requires a vector with single 5' thymidine overhang, provided by the system. For correct cloning the exogeneous DNA fragments must have single 3' adenine overhang. This is obtained by the RT-PCR/PCR reaction used for gene isolation from the viral RNA genome. By adding a Taq DNA polymerase treatment, the single 3' adenine overhang was optimalized. Positive transformants was easily detected by the bacterial coloration though a further analysis with restriction digestion was needed to definitely characterize positive recombinant plasmids. We have isolated positive recombinant bacterial colonies, harvesting pCR2.1-TOPO plasmid with *env-tm* gene insertion. This cloning procedure constitutes the first step for the expression of *env-tm* protein in *E. coli* or in eukaryotic cells. Cloning in pCR2.1-TOPO constitutes an additional step. Nevertheless, thanks to the cloning efficiency, the gene isolation was attained in a much shorter time. Finally, the

intermediary construct will also allow correct excision of *env-tm* gene so that the insertion in a prokaryotic as well as in an eukaryotic expression vector will be done in oriented manner and conserving the gene reading frame.

Acknowledgment

This work was supported by Directorate General of High Degree, The Ministry of National Education. No. 020/LIT/BPPK-SDM/IV/2002.

References

Chadwick, B. J., R. J. Coelen, L. M. Sammels, G. Kertayadnya, and G. E. Wilcox. 1995a. Genomic sequence analysis identifies Jembrana disease virus as a new bovine lentivirus. J. Gen. Virol., 76: 189-192.

Chadwick, B. J., R. J. Coelen, G. E. Wilcox, L. M. Sammels, and G. Kertayadnya. 1995b. Nucleotide sequence analysis of Jembrana disease virus: a bovine lentivirus associated with an acute disease syndrome. J. Gen. Virol., 76: 1637-1650.

Chadwick, B. J., M. Desport, J. Brownlie, G. E. Wilcox, and D. M. Dharma. 1998. Detection of Jembrana disease virus in spleen, lymp nodes, bone marrow and other tissues by *in situ* hybridization of paraffin-embedded secondstions. J. Gen., Virol., 79: 101-106.

Chen, H., J. He, S. Fong, G. Wilcox, and C. Wood. 2000. Jembrana disease virus Tat can regulate human immunodeficiency virus (HIV) long terminal repeat-directed gene expression and can substitute for HIV Tat in viral replication. J. Virol., 74: 2703-2713.

Hartaningsih, N., G. E. Wilcox, D. M. Dharma, and M. Soetrisno. 1993. Distribution of Jembrana disease in cattle in Indonesia. Vet. Microbiol., 38: 23-29.

Metharom, P., S. Takyar, H. H. Xia, K. A. Ellem, J. Macmillan, R. W. Shepherd, G. E. Wilcox, and M. Q. Wei. 2000. Novel bovine lentiviral vector based on Jembrana disease virus. J. Gene Med., 2: 176-185.

Soeharsono, S., G. E. Wilcox, D. M. Dharma, N. Hartaningsih, G. Kertayadnya, and A. Budiantono. 1995a. Species differences in the reaction of cattle to Jembrana disease virus infection. J. Comp. Pathol., 112: 391-402.

Soeharsono, S., G. E. Wilcox, A. A. Putra, N. Hartaningsih, K. Sulistyana, and M. Tenaya. 1995b. The transmission of Jembrana disease, a lentivirus disease of Bos javanicus cattle.[4] Epidemiol. Infect., 115: 367-374.

Wilcox, G. E., B. J. Chadwick, and G. Kertayadnya. 1995. Recent advances in the understanding of Jembrana disease. *Vet. Microbiol.*, 46: 249-255.

Wilcox, G. E., G. Kertayadnya, N. Hartaningsih, D. M. Dharma, S. Soeharsono, and T. Robertson. 1992. Evidence for a viral aetiology of Jembrana disease in Bali cattle. Vet. Microbiol., 33: 367-374.